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Short communication

Hybridization assay of insect antifreezing protein gene by novel multilayered porous silicon nucleic acid biosensor

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ABSTRACT

A fabrication of a novel simple porous silicon polybasic photonic crystal with symmetrical structure has been reported as a nucleic acid biosensor for detecting antifreeze protein gene in insects (*Microdera puntipennis dzhungarica*), which would be helpful in the development of some new transgenic plants with tolerance of freezing stress. Compared to various porous silicon-based photonic configurations, porous silicon polytype layered structure is quite easy to prepare and shows more stability; moreover, polybasic photonic crystals with symmetrical structure exhibit interesting optical properties with a sharp resonance in the reflectance spectrum, giving a higher *Q* factor which causes higher sensitivity for sensing performance. In this experiment, DNA oligonucleotides were immobilized into the porous silicon pores using a standard crosslink chemistry method. The porous silicon polybasic symmetrical structure sensor possesses high specificity in performing controlled experiments with non-complementary DNA. The detection limit was found to be 21.3 nM for DNA oligonucleotides. The fabricated multilayered porous silicon-based DNA biosensor has potential commercial applications in clinical chemistry for determination of an antifreeze protein gene or other genes.

1. Introduction

Although Xinjiang is an important high-quality long staple cotton production base in China and even in the world, low-temperature damage has hindered the increase in cotton production. However, the conventional breeding methods cannot easily improve the cold tolerance of cotton and other plants, thus biotechnology (transgenic technology) has been studied to breed new cotton varieties, such as transgenic cotton using an antifreezing protein (AFP) gene from some hardy animals and plants. Many studies have shown that AFP from insects has specific activities 10–100 times greater than most AFPs from other organisms at comparable concentrations (Yan et al., 2008). In our laboratory, the AFP gene has already been isolated from *Microdera puntipennis dzungarica*, a beetle in the Xinjiang desert region to protect transgenic plants from cold or freezing damage (Yan et al., 2008). Throughout the breeding process, the detection of AFP genes from desert insects plays a fundamental role. The conventional methods for quantitatively determining DNA sequences, such as Southern blot, ELISA and PCR–ELISA, are sensitive but require labeling with a fluorescent probe. Also, they have numerous disadvantages; for example, they are time-consuming and suppress specific hybridization. Therefore development now tends toward label-free and miniaturized techniques (Chunyan et al., 2008; Tatsuro et al., 2005).

Recently, some label-free detection systems using biosensors have been suggested, and have become increasingly popular and important in the determination of oligonucleotide hybridization (Tatsuro et al., 2005). Porous silicon (PSi) is an attractive material for label-free biosensors, showing many worthy properties which make it suitable as a sensing transducer (Guoguang et al., 2008a). Various complicated PSi-based configurations have emerged as optical biochemical sensors, such as Bragg mirrors, waveguides, grating waveguides, diffraction gratings, and so on (Guoguang et al., 2008a; Judson et al., 2010; Xiaoyi et al., 2009; Xing et al., 2011). Nevertheless, the most widely used PSi-based photonic design is the multilayered PSi (so-called PSi photonic crystal) due to its ease of testing, lower cost and ease of preparation by periodically altering the etching current. By contrast, for example, PSi grating relies on relatively expensive lithography and

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PSi waveguides need a rutile prism for detecting coupled light. Among these multilayered PSi, some types following a novel sequence exhibit excellent features for sensing applications, such as Thue-morse and Cantor multilayer (Escorcia-García et al., 2012; Luigi et al., 2007). However, there are many external random fluctuations produced by the electrochemical process in the fabrication of PSi photonic crystal, which have a great influence on the fabrication of accurate PSi photonic crystal and limit its commercial application. To address this issue, in our previous work, we demonstrated a novel simple PSi polybasic Bragg mirror following the $(n_1n_2n_3)^m$ sequence with more stability and smaller influence of disorder, where n_1, n_2 and n_3 represent different refractive index layers (Xiaoyi et al., 2011). Although more stable, the broad peak in the reflectance spectrum of the polybasic Bragg mirror gives a low-Q (which is defined as $Q=\lambda/\Delta\lambda$ and the sensitivity increases with the Q factor), which has hindered the development of higher-performance sensors (Huimin et al., 2005). Li et al. demonstrated theoretically that one-dimensional photonic crystal made of three kinds of dielectric mediums with symmetrical structure shows a resonance dip in the reflectance spectrum, which means this structure gives a high value of the Q factor (Li et al., 2007).

Inspired by the distinctive optical properties of polybasic photonic crystal with symmetrical structure and our previous work cited above, in this paper, we report the demonstration of a novel simple PSi polybasic structure following the $(n_1n_2n_3)^m(n_3n_2n_1)^m$ sequence with more stability and higher sensitivity as a nucleic acid biosensor. DNA hybridization of the AFP gene from *M. puntipennis dzungarica* was for the first time directly detected by a label-free PSi biosensor, helpful for quickly raising cold-tolerant cotton. This research also lays the foundation for developing a sensitive label-free biosensor for the monitoring of other biomolecules.

2. Materials and methods

2.1. Reagents

3-aminopropyltriethoxysilane (APTES), HEPES buffer, glutaraldehyde, sodium cyanoborohydride and ethanolamine for chemical modification of PSi were purchased from Aladdin Reagent Co. (China, www.aladdin-reagent.com). All the chemicals were of analytical reagent grade.

Three DNA oligonucleotides were prepared by the key laboratory of Xinjiang biological resources and gene engineering and the sequences related to AFP gene from *M. puntipennis dzungarica* were shown:

Probe DNA: 5′-AACTGCAATAGAGCGATGACGTG-3′ (23-base);

Complementary DNA: 5′-CACGTCATCGCTCTATTGCAGTT-3′ (23-base);

Non-Complementary DNA: 5′–TTGGACATCCTGTTGAATGAGTGC-3′ (24-base).

2.2. PSi structure

Fig. 1a shows a schematic cross-section of the proposed novel simple PSi polybasic structure following the $(n_1n_2n_3)^m(n_3n_2n_1)^m$ sequence, where n_1, n_2 and n_3 represent different refractive index layers, respectively; and m is the number of periods. In our previous work, a PSi polybasic multilayer following the $(n_1n_2n_3)^m$ sequence was demonstrated to effectively lessen the impact of the disturbance from electrochemical etching and in Li et al. (2007) the authors demonstrated theoretically that there could be a narrow peak of one-dimensional photonic crystal following the $(n_1n_2n_3)^m(n_3n_2n_1)^m$ sequence in the reflectance spectrum.

A comparison of the reflectance spectra of the PSi $(n_1n_2n_3)^{12}$ sequence and PSi $(n_1n_2n_3)^6(n_3n_2n_1)^6$ sequence was calculated in Fig. 1b using the transfer matrix method and the measured reflectance spectra of PSi $(n_1n_2n_3)^6(n_3n_2n_1)^6$ sequence multilayer shown in the inset of Fig. 1b, herein, a reflectivity band centered around 1 μm due to the low values of absorption coefficients in the infrared region of PSi. Following our earlier work, the refractive indices n_1, n_2, and n_3 were determined by experiment to be 1.8, 1.5 and 1.2, respectively, by using Bruggemann's effective medium theory. The layer thicknesses that correspond to the refractive indices are 120 nm, 100 nm, and 120 nm respectively. We can see clearly from Fig. 1b that a sharper peak is exhibited in the reflectance spectra of the symmetrical structure compared with the normal sequence structure and the calculated reflectance spectra shows good agreement to the experimental data (inset of Fig. 1b). In the $(n_1n_2n_3)^6(n_3n_2n_1)^6$ structure, two layers of n_3n_3 in the center part play a similar role as a defect layer in the microcavity structure. The optical field will be localized in the defect layer, resulting in resonant tunneling which exhibits as a narrow band in the band gap with a higher Q factor. Adjusting only the order of the PSi sequence without changing the refractive index, thickness and number of periods, causes a resonance dip in the reflectance spectrum, which means this structure gives higher sensitivity than the PSi $(n_1n_2n_3)^m$ sequence due to a high value of the Q factor. Thus, PSi photonic crystal with a symmetrical structure gives a higher value of the Q factor while still retaining the stability of a normal PSi polybasic sequence structure.

2.3. Fabrication

The PSi photonic crystal samples were prepared with highly p-type silicon ($\rho=0.01\ \Omega$ cm) by electrochemical etching in an electrolyte solution (49%HF: ethanol=1:1, in volume) and PSi polybasic multilayer with symmetrical structure was fabricated following the $(n_1n_2n_3)^6\,(n_3n_2n_1)^6$ sequence. The different refractive index layers were obtained by using Labview to alternate the anodization current for different etching times. The n_1 layers were formed at a current density of 20.0 mA/cm^2 for 4 s, n_2 at 40.0 mA/cm^2 for 3 s, and n_3 at 80.0 mA/cm^2 for 2.5 s with a 10 s pause after each layer formation.

2.4. Functionalization and optical data acquisition

After anodization, all the freshly etched sensors were oxidized by treating with H_2O_2 for 10 h to form a silica-like internal surface as a necessary step for the subsequent biosensing functionalization (Juankun et al., 2007). After this treatment, the samples were treated with standard coupling chemistry by using aminopropyltriethoxysilane (APTES) and glutaraldehyde and the complete details of the process in this work were performed strictly in accordance with the experimental procedures in Guoguang et al. (2008a).

The average pore size of PSi is about 20 nm in diameter in this research, as in Guoguang's work (Guoguang et al., 2008a); thus 23-base or 24-base oligonucleotides (probe DNA, complementary DNA and non-complementary DNA) were prepared in the same size as Guoguang et al. (2008a). After each step, the reflectivity spectra of the sample were taken by UV–vis spectrophotometer (Hitachi U4100, Japan) with a resolution of 0.1 nm. SEM and TEM images of the multilayered porous silicon nucleic acid sensors were measured with a Field Emission Scanning Electron Microscope (FESEM) (ZEISS SUPRA55 VP, Germany) and scanning transmission electron microscopy (STEM) was conducted at 200 kV with a JEM-2010F field emission TEM (see Supplementary material).

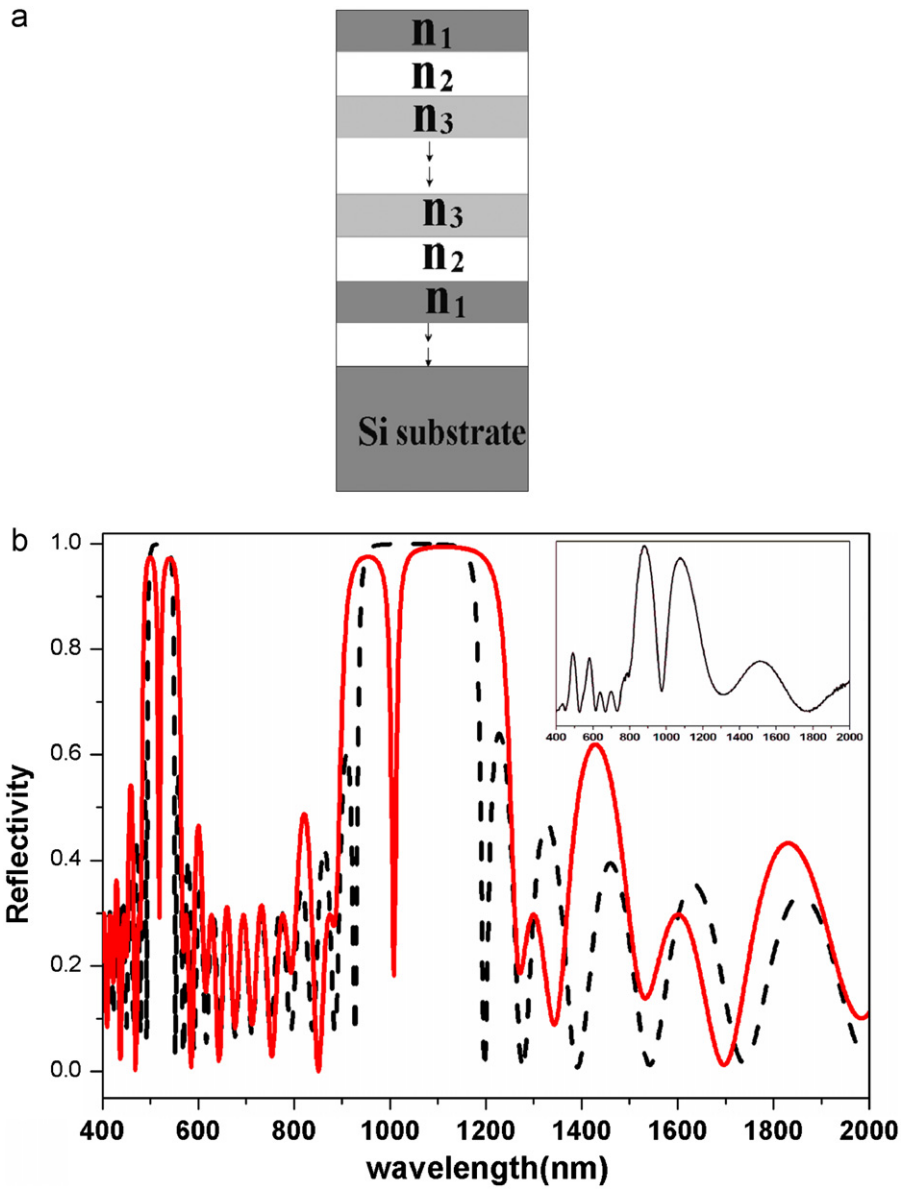

Fig. 1. (a) Schematic cross-section of PSi polybasic multilayer with symmetrical structure. (b) Calculated reflectance spectra of PSi polybasic structure following the $(n_1n_2n_3)^6(n_3n_2n_1)^6$ sequence (solid line) and $(n_1n_2n_3)^{12}$ sequence (dashed line). Inset is the measured reflectance spectra of PSi polybasic structure following the $(n_1n_2n_3)^6(n_3n_2n_1)^6$ sequence.

3. Results and discussion

Fig. 2a shows reflectance spectra of PSi photonic crystal with symmetrical structure after oxidation, after silanization and after glutaraldehyde treatment. It clearly demonstrates that the functionalization steps by APTES and glutaraldehyde produce the red-shift of the reflectance spectrum, which are attributed to an increase in the refractive index of the PSi layer (Huimin et al., 2005) due to the coupling of the small organic molecules, denoting that the functionalization is successful. The reflectance spectra before and after 50 μM of complementary DNA attachment are shown in Fig. 2b and the shift of 83 nm in the spectra due to the specific binding of probe DNA to complementary DNA is reported. To demonstrate specificity, control experiments were performed by using non-complementary DNA and negligible shift in spectra was detected after immersion in 50 μM of non-complementary DNA (Fig. 2c). Additionally, there is no detectable shift after exposure to solutions containing only HEPES buffer (Fig. 2d). Hence, this indicates that red-shift is due to selective DNA hybridization.

Fig. 3a shows the experimental red-shift of the reflectance spectrum as a function of the concentration of complementary DNA. The red-shift is caused by the probe DNA and complementary DNA binding in the pores of the PSi photonic crystal, followed by an increase of the refractive index. The linear fit of the experimental data in Fig. 3a provides the sensitivity of the sensor, which is 4.69 nm/μM. As the resolution of the device is 0.1 nm, the lowest detection limit of the biosensor is 0.1/4.69 = 21.3 nM. The computational method of the detection limit was adopted following the literature (Guoguang et al., 2008b). The reproducibility of the DNA sensor was also good, with the experiments in this work undertaken in triplicate between 3 sensors prepared under the same condition. Standard deviations and the coefficient of variation are given in the supplementary material (Table 1S), and the obtained results were compared. Our subsequent experiments will focus on the reusing of the sensor, and how to improve its reproducibility and consistency.

Since the slope of the shift data points represents the sensitivity of the biosensor upon the change of the pore refractive

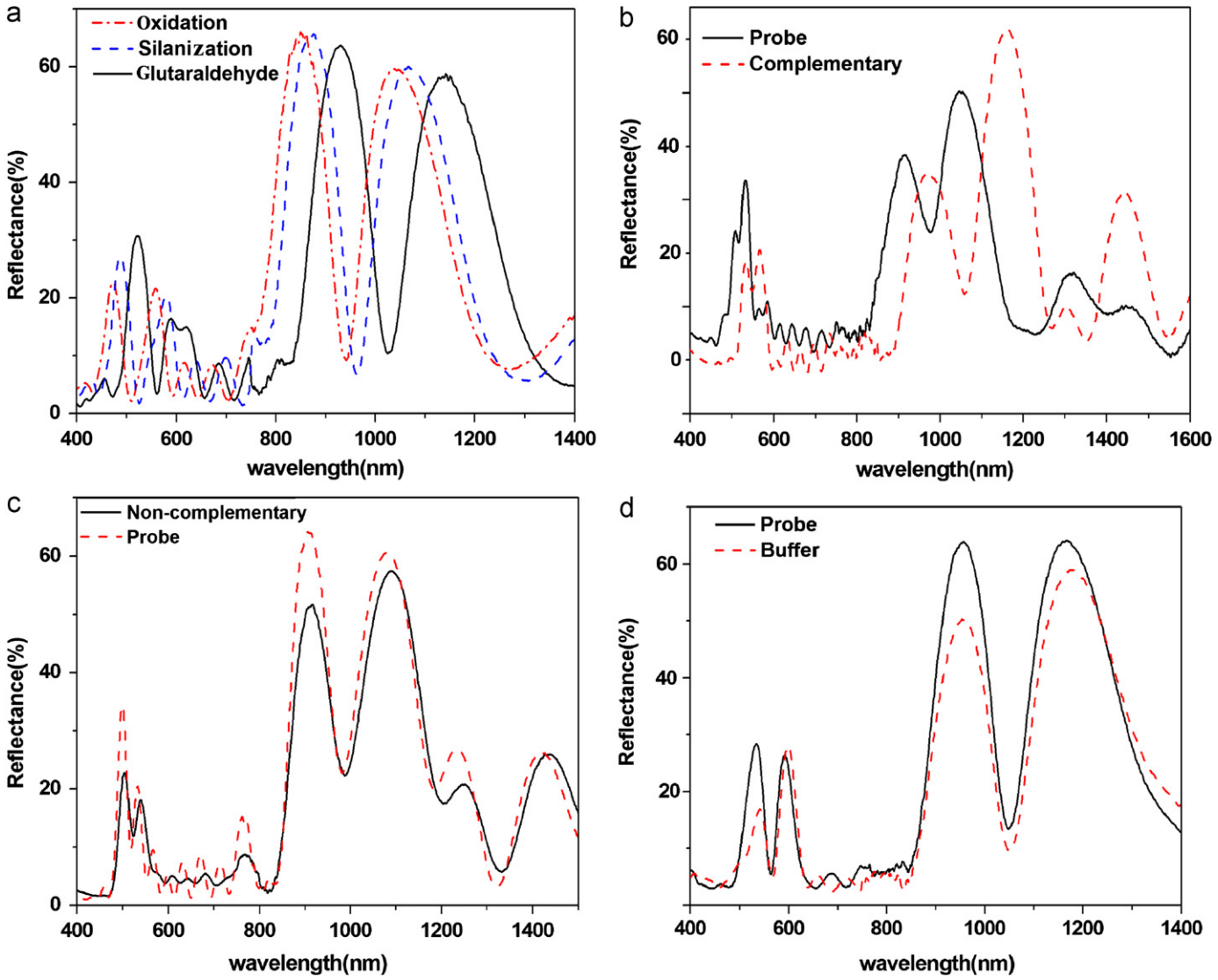

Fig. 2. (a) Reflectance spectra of PSi photonic crystal with symmetrical structure after oxidation, after silanization and after glutaraldehyde treatment. (b) Resonance shift of PSi photonic crystal with symmetrical structure functionalized with complementary DNA (50 μM). (c) Negligible resonance shift for non-complementary DNA (50 μM). (d) No resonance shift for buffer solution.

index caused by the DNA hybridization, Fig. 3b shows the simulated resonance shifts upon refractive index changes in the same way as Guoguang (2009). Somewhat differently, the relative wavelength shift, $\Delta\lambda/\lambda_r$ (λ_r=1010 nm) was employed as a function of refractive index changes as described in Luigi et al. (2007) of TMM sequence PSi multilayer optical sensors. Hence, the detection sensitivity of PSi photonic crystal with symmetrical structure is approximately 0.74 RIU^{-1} (refractive index units), which is higher than the sensitivity of about 0.5 RIU^{-1} in Luigi et al. (2007) with TMM sequence. While sensitivity of the PSi multilayer reported in Luigi et al. (2007) obtained by experiment response of chemical compounds and the number of PSi layers of 64 is more than 36 in our work, it was still shown that the PSi photonic crystal with symmetrical structure exhibits good performance as biochemical sensor.

Even though the DNA hybridization detection limit of the nM concentration was less sensitive as compared with PCR or some other novel method like rutin–Cu nucleic acid biosensor with limit of 2.3 nM 24-base oligonucleotides (Shu-Yan et al., 2009), our biosensor is simple, label-free, low cost and relatively selective. Also, the PSi polybasic symmetrical structure sensor has sensitivity better than or comparable to other PSi-based configuration biosensors. Experiments are in progress to optimize the parameter and enhance the effectiveness for DNA hybridization detection. Furthermore, PSi multilayered films with polybasic symmetrical structure can be encoded for biomolecular screening due to their stability, the smaller effect of the random fluctuation on etching, narrow reflectivity features and also the control of the center wavelength by the etching parameters (Frédérique et al., 2002).

4. Conclusions

We have successfully experimentally fabricated and demonstrated a sensitive label-free nucleic acid biosensor based on a novel PSi polybasic symmetrical structure for detecting AFP gene from insects (*M. puntipennis dzhungarica*). Novel PSi polybasic photonic crystal with symmetrical structure appears to be a stable and sensitive tool for the detection of DNA hybridization. As a biosensor platform, our sensor is label-free and simple, has good selectivity and shortens the detection time and cost

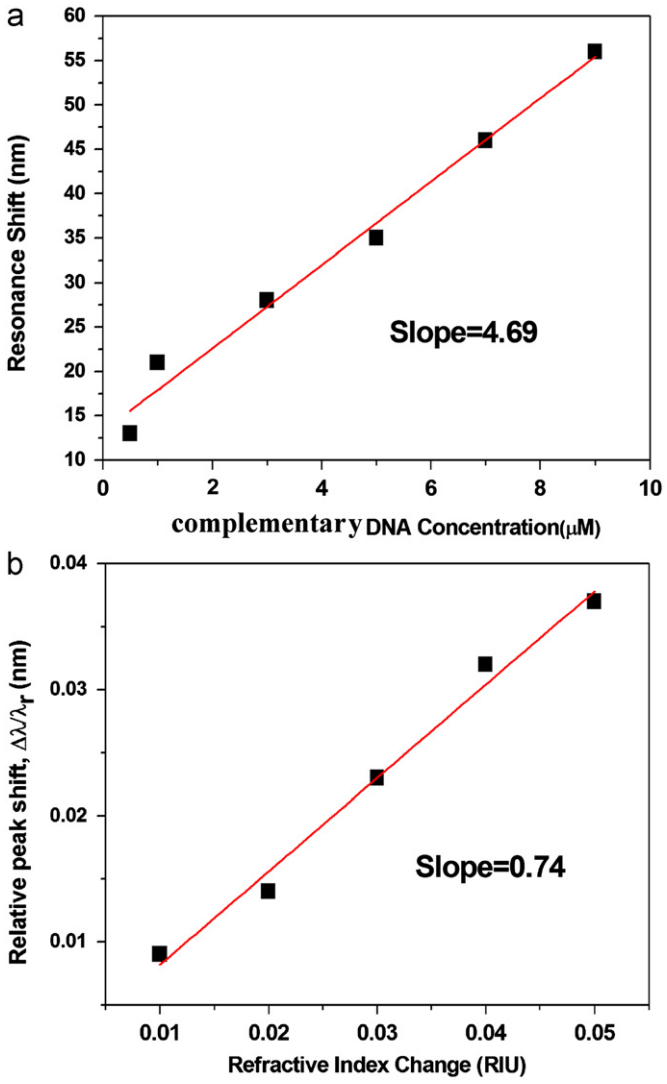

Fig. 3. (a) Measured resonance shift of PSi photonic crystal with symmetrical structure after exposure to various concentrations of complementary DNA. (b) Simulated resonance shift upon refractive index changes of the PSi photonic crystal with symmetrical structure. The slope of the linear fit represents the sensitivity.

compared to other biosensor techniques (such as electrochemical, PCR, and PSi waveguide). In short, this platform not only offers the advantages both of PSi and photonic crystal sensors, but also effectively lessens the impact of the disturbance from etching and shows a high value of the *Q* factor with high sensitivity. Thus, the developed novel PSi nucleic acid biosensor would be helpful for quickly raising cold-tolerant cotton and also might have an immense potential in clinical chemistry for rapid and convenient determination of DNA hybridization.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.07.047.

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